Nerve Growth Factor Prevents the Apoptosis-associated Increase in Acetylcholinesterase Activity after Hydrogen Peroxide Treatment by Activating Akt

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Abstract  Acetylcholinesterase (AChE) is thought to play an important role during apoptosis. Our results showed that H2O2 induced AChE activity, a functional marker in apoptosis, increases in neuronal-like PC12 cells. Glutathione, which is involved in cellular redox homeostasis, inhibited the increase of AChE activity, suggesting that reactive oxygen species (ROS) play a key role in this process. Further investigation showed that the elevation of AChE was observed after the degradation of Akt, release of cytochrome c from mitochondria into the cytosol, and activation of caspase family members. When nerve growth factor (NGF) was present, with the maintenance of Akt level, the elevation of AChE, the cytochrome c diffusion, as well as apoptosis were markedly attenuated in H2O2-treated PC12 cells. However, wortmannin, an inhibitor of the PI3K/Akt pathway, accelerated the apoptosis and increased the AChE activity. The overexpression of constitutively activated Akt, which is a downstream signalling element of the NGF receptor TrkA, delayed mitochondrial collapse and inhibited elevation of AChE activity. Thus, NGF prevented apoptosis and elevation of AChE activity by activating the Akt pathway and stabilizing the function of mitochondria.

Key words  acetylcholinesterase (AChE); reactive oxygen species (ROS); Akt; apoptosis

In the nervous system, apoptosis is required for normal development [1] and is also implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer’s disease (AD) and Parkinson’s disease [2]. Acetylcholinesterase (AChE) aggregates in the senile plaques of the brains of AD and the cholinergic dysfunction in AD have been attributed to stress-induced increases in AChE activity [3, 4]. Although AChE is expressed during apoptosis of cell lines of different origins, the exact mechanism by which AChE executes the apoptotic function is still not known [5]. Melo et al. reported that oxidative stress enhanced AChE activity in cultured cells [6]. Park et al. proposed that AChE was involved in apoptosome formation in apoptosis [7], which is one of the steps in caspase activation.

The AChE inhibitors huperzine A and tacrine were able to attenuate H2O2-induced apoptosis in PC12 cells [8,9]. Although the survival effects were due to the regulation of expression of apoptosis-related genes, the possibility also exists that AChE inhibitors protected cells by binding AChE and influencing the function of this enzyme.

Neurons are particularly sensitive to oxidative damage. Neurotrophic factors such as nerve growth factor (NGF) are essential for neuronal survival. NGF binds and activates the cell membrane-associated NGF receptor TrkA [10]. Rat pheochromocytoma PC12 cells, which have the NGF receptor TrkA in the cell membrane and are responsible to NGF stimulation, are extensively used to study the differentiation and apoptosis of neurons [11]. This cell displayed typical neurite outgrowth and the differentiation marker AChE activity increased when cultured with NGF [12]. However, with a high AChE level, the differentiating
PC12 cells induced by NGF did not display a higher apoptotic rate. The same status was also observed in neurons and cultured cells with background AChE expression. The predominant mechanism involved in NGF-mediated cell survival includes activation of the phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B cascades, which are the signaling pathways downstream of TrkA [13]. Akt is phosphorylated by PI3K at Thr308, as well as the C-terminal Ser473 [14]. Activated Akt preserves the mitochondrial integrity by phosphorylating the pro-apoptotic Bcl-2 family member Bad [15]. Cytochrome c is located in the inner mitochondrial membrane and is released into the cytoplasm during apoptosis when mitochondrial integrity is broken [16]. The released cytochrome c binds procaspase-9 and Apaf-1, as well as dATP to activate caspase-9 [17]. Therefore, Akt prevents the activation of caspase-9 and its downstream caspasers, such as caspase-3, upstream of cytochrome c [18].

In this report, we examined the apoptosis-associated AChE change in PC12 cells. By exposing PC12 cells to H2O2, incubating PC12 cells with NGF, overexpressing constitutively activated Akt, carrying out the cellular assays of apoptosis and examining the AChE activity, we reported that the AChE activity increase was observed before the increase in AChE. NGF prevented this increase by phosphorylating Akt, explaining how NGF protects cells.

**Material and Methods**

**Reagents**

Rabbit polyclonal anti-cleaved caspase-3 antibodies, rabbit polyclonal anti-Akt antibodies, and mouse monoclonal anti-phosphorylated Akt (Ser473) antibody were purchased from Cell Signaling Technology (Beverly, USA). Mouse monoclonal anti-β-actin antibody was purchased from Sigma (St. Louis, USA). The mouse monoclonal anti-cytochrome c antibody clone 6H2.B4 was purchased from Promega (Madison, USA). Horseradish peroxidase (HRP)- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). RPMI 1640 medium was purchased from Invitrogen Corporation (Grand Island, USA). NGF and caspase inhibitor z-VAD were purchased from Promega. Wortmannin, dimethylsulfoxide (DMSO), Hoechst 33258, RNase A and proteinase K were from Sigma. The transfection reagent Lipofectamine 2000 was from Invitrogen.

**Cell culture and transfection**

PC12 cells (passage 10–20) were cultured in RPMI 1640 medium with 5% newborn calf serum, 10% horse serum, streptomycin (100 μg/ml) and penicillin (100 U/ml) at 37 ºC in 95% air and 5% carbon dioxide. For each experiment, PC12 cells were plated in RPMI 1640 medium at a specific density the day before the experiment was carried out. On the day of the experiment, the culture medium was replaced with fresh serum-free medium prior to addition of H2O2.

**DNA fragmentation assay**

For DNA fragmentation, cells (2×10⁶) were collected, washed once with phosphate-buffered saline (PBS), and fixed with 70% ethanol overnight at −20 ºC. Only small DNA fragments were extracted according to the method of Gong et al. [19]. Briefly, DNA was extracted with 0.2 M phosphate-citrate buffer at pH 7.8 for 1 h at room temperature, treated with RNase A and proteinase K, and then subjected to electrophoresis.

**AChE catalytic activity determination**

Relative AChE activity was determined spectrophotometrically in a 96-well plate using a modified Ellman’s assay as described [20]. Cells were suspended in extraction buffer (50 mM potassium phosphate, pH 7.4, 1 M NaCl, 0.5% Tween-20). Lysates were clarified by centrifugation for 10 min at 4 ºC, and the protein concentration of the supernatants was assayed using a Biochimniconic acid (BCA) method. Relative AChE activity was measured with an equal amount of total protein. Tetraitsoprolpy pyrophospho-reonicle (iso-OMPA, 0.75 mM) was added to exclude possible BuChE activity. The AChE activity was expressed as multiple relative to untreated control.
Cytochrome c immunocytochemical staining

Cells were seeded on slides and treated as indicated. After fixation with paraformaldehyde (4% in PBS), cells were permeated with 0.1% Triton in 0.1% sodium citrate solution on ice and washed three times with Tris-buffered saline with Tween (TBS-T) (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20). Cells were incubated with 0.5 ml TBS-T containing 5% bovine serum albumin (BSA) as blocking buffer for 40 min at room temperature and then incubated with 50 μl primary antibody (1:100 dilution in TBS-T containing 5% BSA) for 1 h at 37 °C. After washing three times with TBS-T, cells were incubated with 50 μl secondary antibody (1:100 dilution in TBS-T containing 5% BSA) for 1 h at 37 °C. Cells were again washed with TBS-T and 5 μM Hoechst 33258 was used to stain the nuclei. The cells were then examined under a Leica TCS SP2 confocal immunofluorescence microscope (Mannheim, Germany).

Western blotting

For Western blotting, equal amounts of total protein were separated using a sodium dodecyl sulfate-polyacrylamide gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with TBS-T containing 5% fat-free dried milk for 2 h at room temperature and further incubated with a primary antibody overnight at 4 °C. Afterwards, the membrane was washed with TBS-T three times and incubated with an HRP-conjugated secondary antibody for 2 h at room temperature. After the membrane was washed again, the immunoreactive protein was visualized using the enhanced chemiluminescent reagent ECL (Santa Cruz Biotechnology, Santa Cruz, USA) according to the manufacturer’s protocol.

Statistical analyses

All experiments were repeated at least three times. Statistical analyses were carried out using the Student’s two-tailed t test with \( P<0.05 \) considered to be significant.

Results

H₂O₂ induced the increase in AChE activity in apoptotic PC12 cells

PC12 cells were exposed to varying concentrations of H₂O₂ (0–1 mM) for 30 min and fragmented DNA was extracted to determine the extent of cell apoptosis after 24 h. The results showed that H₂O₂ induced apoptosis at concentrations between 100 μM and 1 mM. Apoptosis was evaluated by the generation of a characteristic “ladder” pattern of discontinuous DNA fragments. As shown in Fig. 1(A), 100 μM H₂O₂ induced a typical pattern of DNA laddering fragmentation. AChE activity was also measured by a modified Ellman’s assay. Fig. 1(B) shows that 100 μM H₂O₂ resulted in an (80±20)% increase in AChE activity, which correlated to apoptosis as indicated by discontinuous DNA fragments. Further increasing the concentration of H₂O₂ from 200 μM to 1 mM did not induce any significant increase in AChE activity. The results indicated that H₂O₂ induced apoptosis and an almost doubled increase in AChE activity. Therefore, we treated cells with 100 μM H₂O₂ in subsequent experiments. We also did a time-course of AChE activity up to 36 h after treatment with 100 μM H₂O₂. The increase in AChE activity was first observed by

Fig. 1  H₂O₂-induced apoptosis and parallel increase in acetylcholinesterase (AChE) activity in PC12 cells

(A) H₂O₂ at concentrations of 100 μM or more induces DNA laddering. PC12 cells were treated with 0–1000 μM H₂O₂ for 30 min and cultured in serum-free medium, and after 24 h, soluble DNA was extracted to measure DNA fragmentation. Data were from a representative experiment carried out three times. (B) PC12 cells were treated as described above and the relative AChE activity was measured. (C) PC12 cells were treated with 100 μM H₂O₂, as described above, collected every 12 h up to 36 h, and AChE activity was measured. Data were represented as mean±SD of three independent experiments.

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12 h after the H₂O₂ treatment, peaked by 24 h, and remained stable to 36 h [Fig. 1(C)].

Glutathione attenuated H₂O₂-induced apoptosis and increase in AChE activity

Hydrogen peroxide was reported to initiate the apoptotic cascade by perturbing the intracellular redox balance [21]. GSH is the most abundant low molecular weight thiol that maintains cellular redox homeostasis [22]. To determine whether the increase in AChE activity was associated with alterations in H₂O₂-induced cellular redox status, we pretreated and then cultured cells with 5 mM GSH. As indicated in Fig. 2(A–C), the presence of GSH protected the cells against H₂O₂-induced apoptosis and the increase in AChE activity. Therefore, the redox imbalance resulted in mitochondrial dysfunction and enhanced mitochondrial reactive oxygen species (ROS) generation.

NGF and phosphorylated Akt prevent the increase in H₂O₂-induced AChE activity

The NGF protected neuronal cells from oxidative stress by activating the PI3K and Akt signaling pathways [23]. We investigated whether NGF was able to eliminate the H₂O₂-induced increase in AChE activity by maintaining Akt phosphorylation. We first detected the phosphorylated Akt level by Western blotting with an anti-phosphospecific Akt (Ser473) antibody. The total Akt level was detected with an anti-Akt antibody. To further investigate whether Akt de-phosphorylation was related to the increase in AChE activity, we introduced the PI3K inhibitor wortmannin. We pretreated cells with 100 nM wortmannin for 30 min before treating cells with H₂O₂. Wortmannin was also present in the serum-free medium after H₂O₂ treatment. For NGF, 50 ng/ml of NGF was incubated with PC12 cells before and after H₂O₂ treatment. As shown in Fig. 3(A), 30 min of H₂O₂ treatment induced a phosphorylation of Akt lasting at least 6 h, although the phosphorylated Akt was barely detectable 12 and 24 h after H₂O₂ treatment. Although less than in untreated cells, Akt was also detected at 24 h in cells after H₂O₂ treatment. Wortmannin accelerated dephosphorylation of Akt, due to phosphorylation Akt in wortmannin-treated cells being abolished by 6 h after H₂O₂ treatment. In contrast, high levels of Akt and phosphorylated Akt were still detected in NGF-treated PC12 cells at 24 h after the H₂O₂ challenge. There appeared to be a delay between loss of Akt activity, as detected by phosphorylation of Ser437, and its proteolytic degradation, similar to the results of Martin et al. [24]. This might because oxidant-induced downregulation of Akt proceeds in two steps: first, inactivation of Akt, and second,
proteolytic degradation.

AChE activity was detected in wortmannin- and NGF-treated cells. Fig. 3(B) shows that AChE activity increased faster in cells treated with H$_2$O$_2$ and wortmannin, compared with cells treated with H$_2$O$_2$ alone. By 6 h, the wortmannin-treated cells had a clear increase in AChE activity. In NGF-treated cells, 50 ng/ml NGF was not enough to increase AChE activity in normal PC12 cells by 24 h; however, NGF prevented apoptosis and an increase in AChE activity in H$_2$O$_2$-treated cells by 24 h [Fig. 3(C)]. The inhibiting effect of NGF on the increased H$_2$O$_2$-induced AChE activity was abolished by wortmannin [Fig. 3(D)]. Cells not treated with NGF showed more apoptosis as indicated by condensed nuclei and less apoptosis was observed in NGF-treated cells [Fig. 3(E)]. Therefore, we have shown that AChE activity does not increase earlier than 12 h after H$_2$O$_2$ treatment [Fig. 1(C)], and that there is a negative relationship between the activity of AChE and phosphorylated Akt.

To further verify the effect of Akt activation is related to the increase in AChE activity, we transfected a constitutively active form of Akt into PC12 cells. The constitutively activated Akt contains a Src myristoylation site at the C-terminal end (MyrAkt) and lacks the pleckstrin homology (PH) domain, therefore MyrAkt exhibits constitutive kinase activity independent of PI3-kinase activity or growth factor stimulation [25]. The expression of constitutively active Akt in transfected PC12 cells was confirmed by using an anti-Akt antibody and an anti-phosphospecific Akt (Ser473) antibody. Compared with
Akt, MyrAkt has a lower molecular weight due to the missing PH domain [Fig. 4(A)]. The transfected PC12 cells were treated with H$_2$O$_2$ as described above, and the protective effect of MyrAkt was assessed by the level of DNA laddering fragmentation. The results showed that the cells with stably activated Akt survived H$_2$O$_2$-induced apoptosis with reduced DNA laddering fragmentation [Fig. 4(B)]. A smaller increase in AChE activity was also detected in constitutively activated Akt-transfected cells when treated with H$_2$O$_2$. MyrAkt did not enhance AChE activity in untreated PC12 cells [Fig. 4(C)].

Cytochrome c is released from mitochondria before the increase in AChE activity

Phosphorylated Akt prevented apoptosis mainly by maintaining the integrity of mitochondria and controlling cytochrome c efflux [18]. Exogenously added GSH maintains cellular redox homeostasis, prevents ROS production, and thereby inhibits ROS-mediated cytochrome c release [16,26]. We therefore examined if cytochrome c was released from mitochondria in parallel with the increase in AChE activity. Immunofluorescent staining of PC12 cells with a cytochrome c antibody provided a staining pattern characteristic of mitochondrial localization. In untreated cells, cytochrome c is located in the mitochondria and was indicated by a punctate staining pattern. H$_2$O$_2$-induced apoptosis of PC12 cells was associated with cytoplasmic shrinkage, chromatin condensation, and cytochrome c release from mitochondria. Cells with released cytochrome c showed a faint, homogeneous staining pattern, apparently because cytochrome c was rapidly degraded once released into the cytoplasm. In apoptotic cells, diffused cytochrome c was found in the nuclei, which was marked with the nuclear dye Hoechst 33258. To investigate if AChE activity increased before cytochrome c diffusion, we measured AChE activity, as well as cytochrome c diffusion between 6 and 24 h after H$_2$O$_2$ treatment. As shown in Fig. 5, about 30% of total cells had diffused cytochrome c by 6 h. AChE activity remained stable at 6 h but had increased by 12 h. However, incubation with 100 nM wortmannin before and after H$_2$O$_2$ treatment accelerated cytochrome c diffusion and an increase in AChE activity. By 6 h after the H$_2$O$_2$ treatment, about 70% of wortmannin-treated cells had diffused cytochrome c and AChE activity had increased by 70%. By 12 h after the H$_2$O$_2$ treatment, almost all of the wortmannin-treated cells had diffused cytochrome c and the AChE activity increase was at a peak of twice the level of untreated cells. The maximum effect of H$_2$O$_2$ alone on AChE activity emerged by 24 h after treatment. Meanwhile, NGF and GSH prevented cytochrome c diffusion and an increase in AChE activity even at 24 h after treatment. These results indicate that cytochrome c release from mitochondria is an upstream event occurring before the increase in AChE activity during H$_2$O$_2$-induced apoptosis of PC12 cells. However, because only a large scale release of cytochrome c promoted the maximum increase in AChE activity, we could not exclude the possibility that some events downstream of cytochrome c were involved in the upregulation of AChE activity.

![Fig. 4](http://www.abbs.info; www.blackwellpublishing.com/abbs)
The increase in acetylcholinesterase activity occurs later than caspase-3 activation

Both caspase-3 activation and an increase in AChE activity occur after the cytochrome c release. To further clarify the time course of the AChE activity increase and caspase-3 activation, we treated cells with H₂O₂ for up to 24 h and measured caspase-3 levels by Western blotting at various times using an anti-cleaved caspase-specific antibody. Although slight caspase-3 activation was detected by 5 h after H₂O₂ treatment, the increase in AChE activity did not occur earlier than 12 h after the H₂O₂ incubation [Fig. 1(C) and Fig. 6(A)]. Therefore, the increase in AChE activity occurred after caspase was activated in a larger scale. The broad-spectrum caspase inhibitor z-VAD-fmk was used to verify the impact of caspase-3 activation on the Akt level and the AChE increase after H₂O₂ treatment. The addition of z-VAD-fmk to the culture medium at a final concentration of 50 μM resulted in a decrease in caspase-3 activation and an increase in AChE activity. Furthermore, as a substrate of caspase-3, Akt degradation was reserved in z-VAD-fmk incubated cells [Fig. 6(B,C)]. The loss of Akt after caspase-3 activation might be responsible for the AChE activity increase in a rather late

Fig. 5   Cytochrome c release occurs before the increase in acetylcholinesterase (AChE) activity
PC12 cells were incubated with 5 mM glutathione (GSH), 50 ng/ml nerve growth factor (NGF), or 100 nM wortmannin for 30 min before treatment with H₂O₂ for 30 min. Cells were then cultured in serum-free medium with GSH, NGF, or wortmannin for the indicated period. (A) Cytochrome c immunostaining and Hoechst33258 staining of PC12 cells. Fixation and staining of cells was done at the indicated times. Arrows indicate diffuse cytochrome c staining and the corresponding nucleus of the cell. I–III, normal cells; IV–VI, cells treated with H₂O₂+wortmannin for 12 h. (B) The number of cells with diffused cytochrome c staining pattern was counted and expressed as percentage of the total number of cells. Data were represented as mean±SD of the percentage of cells from three independent experiments. At least 200 cells were counted for each sample. (C) Relative AChE activity was measured by the modified Ellman’s assay. Data were represented as mean±SD of three independent experiments.
stage of apoptosis.

Discussion

AChE was reported to express during apoptosis of cell lines of different origins. The AChE activity, which was taken as a functional marker, was found increased during neuron differentiation or cell apoptosis. When cells were exposed to apoptotic stimuli, which included long-term culture and chemotherapy agents, AChE was detected before the appearance of apoptotic morphology [5,7]. So AChE was supposed to play a role in cell apoptosis. However, the AChE change in those cells with background expression of this esterase was poorly illuminated. One fact was verified that AChE would not initiate apoptosis in AChE containing cells, such as PC12 cells, or in AChE overexpressed cells without any apoptotic stimuli [27]. Therefore, we examined the AChE change in the AChE containing PC12 cells in an apoptotic condition and what effect influenced the AChE change. Our results showed that H2O2 induced an AChE increase in apoptotic cells by mechanisms that required ROS production. The neurotrophic factor NGF prevented the apoptosis-associated increase in AChE by maintaining Akt phosphorylation.

Mitochondrial respiration in higher organisms constantly generates low levels of potentially dangerous ROS, which includes superoxide anion. Mitochondrial superoxide dismutases convert superoxide into H2O2, a non-radical molecule that generates highly toxic hydroxyl radicals via the Fenton reaction [28]. This reaction was separated from the cytoplasm by a mitochondrial double membrane. A transient H2O2 treatment is thought to perturb the cellular redox balance, which is maintained by cellular GSH/GSSH status, disrupt the mitochondrial integrity and allow the mitochondrial ROS efflux [26]. Thus, the toxic effect of exogenous H2O2 was amplified. Increasing the cellular GSH restores the cellular redox balance and suppresses the elevated level of ROS and apoptosis [29]. In our current study, we provided evidence that exogenous GSH protects cells from apoptosis and prevents the increase in AChE activity [Fig. 2(A)]. Therefore, in order to restrain an apoptosis-associated AChE increase, it was an original step to maintain the mitochondrial membrane integrity and control the ROS efflux.

Because PC12 cells have background AChE expression, and the apoptosis induced by H2O2 is a rapid process, which was indicated by a complete DNA laddering within 24 h [Fig. 1(A)], we supposed that the reasons for the apoptosis-associated AChE change were flexible. The protein modification, enhancement in protein synthesis and elevation on protein stability probably had a shared contribution to the final AChE activity increase. We observed that the AChE activity increase emerged in a time course later than cytochrome c release and caspase-3 activation, which was regarded as a relatively late stage of PC12 cell apoptosis. In the late stage of apoptosis, the ROS that was released from the mitochondria accompanied the mitochondrial membrane disruption, which was signed by the cytochrome c release, and further destroyed other cytoplasmic membrane. AChE activity was reported to be enhanced by low concentrations of H2O2 [30]. One...
explanation is that mitochondrial efflux H\textsubscript{2}O\textsubscript{2} modifies membrane structure through lipid peroxidation, and therefore contributes to modify the activity of the membrane-bound protein AChE [31]. AChE is synthesized as an inactive precursor and then matures into an active subunit in the endoplasmic reticulum [32]. Thus, the other explanation was that AChE could be exposed to cytoplasm when endoplasmic reticulum was destroyed by H\textsubscript{2}O\textsubscript{2}. At least one protein kinase (protein kinase A , PKA), was reported to increase the AChE activity by phosphorylation at non-consensus sites of this enzyme [33]. The apoptotic stimuli also enhanced the mRNA and protein levels of AChE in cells without background AChE expression, which were mediated by calcium signalling or the c-Jun kinase pathway [5,34,35]. Furthermore, as a glycoprotein, the glycosylation plays a key role in keeping the effective biosynthesis and protein stability of AChE [36]. Altered glycosylated AChE was found in the cerebrospinal fluid (CSF) of AD patients, and was probably responsible for the AChE activity increase in AD [37].

Jin et al. reported that AChE overexpression enhanced the cellular sensitivity to apoptotic stimulation, but did not initiate the apoptosis [27]. AChE was also reported to have a de novo synthesis under relatively gentle apoptotic stimuli, which included long-term culture and chemotherapeutic agent incubation, and played a pivotal role in apoptosome assembly [5,7,38]. However, our study indicated that AChE activity increases later than caspase-3 activation. Considering that AChE was stably expressed in PC12 cells, we can not exclude the possibility that the AChE-associated promotion of apoptosis formation did not depend on de novo synthesis of AChE. Thus, compared with those reported influences of AChE on apoptosis, our results indicate a supplement for AChE change manner in AChE containing cells under rapid apoptotic conditions.

The relationship between Akt phosphorylation maintenance and inhibition of AChE activity increase was notable. Akt was phosphorylated by tyrosine kinases, which include insulin and NGF; stimuli that regulate G-protein-coupled receptors and other activators such as H\textsubscript{2}O\textsubscript{2} and zinc [14]. The short-term effect of H\textsubscript{2}O\textsubscript{2} on PC12 cells is the activation of Akt in a PI3K-dependent manner and the inducing of long-term down regulation of Akt in a caspase-dependent manner [39]. Activation of Akt by H\textsubscript{2}O\textsubscript{2}, might reflect an attempt of the damaged cells to survive the oxidative insult [24]. In our experiments, we demonstrated that the AChE activity increase was parallel with Akt dephosphorylation. Except for a maintaining factor of mitochondrial membrane integrity, Akt was found as a mediator of glycometabolism modulation, which was controlled by insulin and insulin-like growth factor [40]. Recently, a hypothesis was put forward that AD could be “type 3 diabetes” [41]. The emergence of AD pathomorphism was coupled with a reduced level of insulin receptor substrate (IRS) mRNA and phospho-Akt (activated), and increased glycogen synthase kinase-3 beta (GSK-3\textbeta) activity, which was inhibited by phospho-Akt, and amyloid precursor protein mRNA expression [42]. The increased activated GSK-3\textbeta and amyloid-\beta, and the increased expression of AChE genes were found in an in vivo model of AD-type neurodegeneration that was generated by intracerebral injection of streptozotocin in rats [43]. In AD, the typical neuropathological event is apoptosis of cholinergic neurons in senile plaques accompanied by abnormal aggregation of some proteins, of which \beta-amyloid peptide and AChE are the most concerned [3,44]. AChE was thought to participate in the formation of senile plaques by aggregating with \beta-amyloid and enhancing the toxicity of \beta-amyloid, because increased AChE activity is only detected in senile plaques while AChE activity decreases in normal tissues [45–47]. Another hypothesis suggested that in the central nervous system (CNS), the cholinergic system was involved in cognitive functions and was modulated depending on the acetylcholine (ACh) [48]. As a neurotrophic factor, ACh on a certain range of concentration will facilitate the synaptic transmission and protect the neuron from death [49,50]. It has been proposed that the role of AChE activity in the CNS is to modulate the ACh level existing in the extracellular space [51]. Thus we supposed that the neurons with an AChE activity increase in a rather late stage of apoptosis were probably more toxic to their surrounding cells than to themselves. But whether similar AChE changes occurred in the cholinergic neurons remained to be further investigated.

In conclusion, our results indicate that the AChE activity increased in a rather late stage in the apoptotic PC12 cells treated with H\textsubscript{2}O\textsubscript{2}, and ROS production was crucial in this process. The neurotrophic factor NGF prevented the AChE activity increase by maintaining the Akt phosphorylation. These data supplied a supplemented manner for AChE change during apoptosis of AChE-containing cells and might be useful as an apoptotic neuron model in the research of AChE toxicity during AD.

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